All of our constructs were assembled with the Gibson Assembly method. Many of our primers were designed with the help of NEB's Gibson Primer builder, NEBuilder.NEB.com.

All PCRs to perform Gibson assemblies were done with NEB's Q5 HotStart 2x Master Mix.

We often used the following protocol for our PCRs:

- 2.5 µl Primer 1
- 2.5 µl Primer 2
- 1 µl of 1:100 dilution of miniprep template DNA
- 6.5 µl NFW
- 12.5 µl HotStart Master Mix

Many times, we altered the amount of primer, water, and template DNA as necessary to get PCRs and assemblies to work.

We would then run the PCR products on a gel to confirm that they were the correct size, and if so, would perform a DpnI digestion to remove residual template DNA:

- 24 µl PCR product
- 2.7 µl CutSmart Buffer
- 0.5 µl DpnI

After the DpnI reaction, we would either PCR Purify or perform a Gel Extraction, depending on if there were extra multiple bands on the gel that we wanted to remove. We followed these protocols as described by QIAGEN, with the one modification of heating Buffer EB to 60°C before eluting to increase yields.

From here, we proceeded to the Gibson reaction. We used NEB's Hifi DNAAssembly Master Mix for our assemblies. We reduced the reaction volume by half, in order to conserve Master Mix.

Finally, we performed a chemical transformation with NEB's 5-alpha Competent *E. coli* with 2 µl Gibson product.

Gibson Assembly